

B22  
Briefly, outdated human platelets were isolated by differential centrifugation and solubilized in lysis buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, containing 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, and 50 mM octylglucoside). The octylglucoside extract was incubated with 1 ml GRGDSPK-coupled SEPHAROSE 4B® overnight at 4°C. After washing with 15 ml column buffer (same as lysis buffer except it contained 25 mM octylglucoside), bound  $\alpha_{\text{IIb}}\beta_3$  was eluted with 1.7 mM  $\text{H}_{12}$  (2 ml) in column buffer. The  $\text{H}_{12}$  eluate was applied to a SEPHACRYL® S-300 High Resolution column (1.5 x 95 cm), and  $\alpha_{\text{IIb}}\beta_3$  was eluted with 10 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$  and 25 mM octylglucoside.

Unactivated  $\alpha_{\text{IIb}}\beta_3$  was isolated by the method of *Fitzgerald et al., Anal. Biochem.* 151:169-177 (1985), with slight modifications. The flow-through fraction of the GRGDSPK-SEPHAROSE® column was applied to a concanavalin A-Sepharose 4B column (1 x 20 cm). Unbound proteins were washed with 50 ml column buffer, and bound  $\alpha_{\text{IIb}}\beta_3$  was then eluted with 100 mM mannose dissolved in column buffer. Fractions containing  $\alpha_{\text{IIb}}\beta_3$  were further purified on a SEPHACRYL® S-300 High Resolution column.

## REMARKS

Claims 1-4 and 6-24 are pending in this application. Claims 1-3 and 9-23 have been withdrawn from consideration as directed to a non-elected invention. Claims 4, 6-8 and 24 are under examination on the merits. The foregoing amendments to the specification have been made to capitalize and identify certain trademarks recited in the application. A marked up version of the application showing the amendments is attached hereto as Exhibit A.

### 1. Response to Objections

#### a. Objection to the specification for failure to capitalize and include generic material for trademarks used in the specification

At page 2 of Paper No. 14, the specification remains objected to for failure to capitalize and include generic material for trademarks used in the specification. Applicant respectfully traverses the objection. Applicant respectfully submits that the use of the trademarks identified by the Examiner at page 3 of the Office Action mailed August 29, 2001 (Paper No. 10) is

permissible because the trademarks each have definite meaning and have been capitalized by the amendments herein.

The use of a trademark is permitted if the product to which the trademark refers is set forth in such language that its identity is clear and it is distinguished from common descriptive nouns by capitalization. *See* MPEP § 608.01(v). The trademarks in the specification identified by the Examiner each have a fixed and definite meaning, which constitute sufficient identification of the product referred thereto. Furthermore, no physical or chemical characteristic of the products referred to by the trademarks are involved in the invention. In compliance with MPEP § 608.01(v), Applicant has amended the specification to capitalize the trademarks identified by the Examiner at page 3 of Paper No. 10 to distinguish them from other proper nouns. In view of the trademarks having a fixed and definite meaning and the trademarks being capitalized by the amendments herein, Applicant respectfully requests withdrawal of the objection of the specification for failure to capitalize and include generic material for trademarks used in the specification.

## **2. Patentability Arguments**

### **a. 35 U.S.C. § 103(a)**

#### **1) The rejection of claims 4 and 6-8 under 35 U.S.C. § 103(a) as being obvious over Iwamoto in view of Yang and Lin**

At page 3 of Paper 14, claims 4 and 6-8 remain rejected under 35 U.S.C. § 103(a) as allegedly being obvious over Iwamoto *et al.* (hereinafter, "Iwamoto") in view of Yang and Lin *et al.* (hereinafter, "Lin"). Applicant respectfully traverses the rejection. To establish a *prima facie* case of obviousness under 35 U.S.C. § 103, the following three criteria must be met: (i) the cited prior art references when combined must teach or suggest all the claim limitations; (ii) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine the reference teachings; and (iii) there must be a reasonable expectation of success. *See* MPEP § 2142. Applicant respectfully submits that the Examiner has not established a *prima facie* case of obviousness, because there is no reasonable expectation of success.

At page 5 of Paper No. 10, the Examiner characterizes the invention as a method drawn to a method of screening for a modulator of cell migration comprising forming test and control

gel matrices of Cyr61, a suspected modulator of cell migration and one of Matrigel, collagen or fibrin, seeding human fibroblast cells presenting  $\alpha_6\beta_1$  integrin onto the test and control matrices, incubating the fibroblast cells, measuring the levels of migration in the test and control matrices by visual inspection, and comparing the levels of cell migration, whereby a modulator of cell migration is identified by its ability to alter the level of cell migration in the test matrix compared to the control matrix.

A combination of the Examiner's characterization of Iwamoto and Lin at pages 5-7 of Paper No. 10 yields every element of the Examiner's characterization of the claimed invention, except for the use of Cyr61 as a chemoattractant. To support that the prior art discloses that Cyr61 has chemoattractant activity, the Examiner relies on the abstract from the dissertation of Yang. In its entirety, the abstract of Yang states as follows:

The growth of mammalian cells is controlled by polypeptide growth factors. Within minutes of growth factor stimulation, the transcription of a group of genes is rapidly activated without *de novo* protein synthesis. These genes have been termed "immediate early" genes and appear to be the first step in a genetic program for growth. *cyr61* was initially identified as an immediate early gene. Cyr61 is a secreted 379 amino acid protein with 38 cysteines comprising 10% of the protein. Cyr61 is secreted to the extracellular matrix and cell surface where it is rapidly taken up and degraded through the lysosomal pathway. Cyr61 binds heparin-agarose with high affinity, and binds to the extracellular matrix in a non-covalent manner primarily through interactions with heparan sulfated proteoglycans. Cyr61 is a member of a family of proteins that absolutely conserve the 38 cysteines and have a putative signal peptide. Other Cyr61 family members have been shown to be chemoattractants, mitogens and proto-oncogenes. Initial functional analysis of Cyr61 has shown that it can suppress foci formation in NIH 3T3 cells. We have also found a chemoattractant activity in serum stimulated NIH3T3 cells that **may be related to Cyr61. Further work will be aimed at refining our knowledge of how Cyr61 and its family members may function in biological processes.**

(emphasis added). In the Amendment and Response to Paper No. 10, the contents of which are incorporated by reference herein, Applicant asserted that the above language from Yang is merely speculation that Cyr61 may have chemoattractant activity. Applicant further asserted that one of ordinary skill in the art would characterize Yang as the findings are summarized

therein: "further work" is necessary to determine "how Cyr61 and its family members may function in biological processes." At page 3 of Paper No. 14, the Examiner did not find the preceding arguments persuasive, stating that "absent some evidence to the contrary, Yang is disclosing confirmed chemoattractant activity."

For determining obviousness, a prior art reference must be considered in its entirety, including those portions that teach away from the claimed invention. *See* MPEP § 2141.02. As part of a Second Supplemental Information Disclosure Statement, Applicant submits therewith the full text of the dissertation of Yang, from which the Examiner relies solely on the abstract. The chemoattractant activity disclosed by Yang was discovered in cell lysates from serum-stimulated NIH 3T3 cells, which had been **partially** purified using a heparin affinity column. *See* page 80, lines 13-17; page 81, lines 3-6.

Yang discloses that the partially purified cell lysates with chemoattractant activity do contain Cyr61; however, there are also several other putative chemoattractants along with Cyr61. Yang also discloses that Fisp12 is partially purified under the same conditions used to partially purify Cyr61. *See* page 80, lines 19-21. Fisp12 is the human homolog of CTGF, a known chemoattractant for NIH 3T3 cells. *See* page 80, lines 7-8. In view of the co-purification of Fisp12 and Cyr61, Yang describes the potential chemoattractant activity of Cyr61 as follows:

Given the potential presence of Fisp12 in the cell lysates being tested, **it is still uncertain to what extent the chemoattractant activity is due to Cyr61, if any.**

*See* page 92, lines 18-19 (emphasis added). In addition to Cyr61 and Fisp12, Yang discloses that several other immediate early gene products having the potential to act as chemoattractants for NIH 3T3 cells are also present in the cell lysates, such as JE, KC, Tis7 and Fic. *See* page 92, line 20 to page 93, line 2. Yang discloses that it was unknown whether or not the observed chemoattractant activity was due to Cyr61, Fisp12, or other such immediate early gene products. *See* page 93, lines 1-5. Yang summarizes the potential chemoattractant activity of Cyr61 as follows:

It is unclear what may contribute to any of these [chemoattractant] activities and only further experimentation will determine if Cyr61 is responsible for some of it.

*See* page 93, lines 8-10.

Taking into account the full disclosure of Yang, one of ordinary skill in the art would have been unsure whether the observed chemoattractant activity was due solely to Fisp12 or Fisp12 in combination with one or more of the many other early gene products present in the cell lysates of serum-stimulated NIH 3T3 cells. The Examiner is correct to note that obviousness does not require absolute predictability; however, at least some degree of predictability is required. *See* MPEP § 2143.03. The disclosure of Yang does not provide the requisite degree of predictability because it is totally uncertain which single immediate early gene product or combination thereof present in the partially purified lysate may be responsible for chemoattractant activity in addition to Fisp12. Yang is at best an invitation to try to determine whether Cyr61, among several other gene products with identified or potential chemoattractant activity, is in fact a chemoattractant. It is the discoveries set forth in the present application that for the first time demonstrate that Cyr61 is a chemoattractant. However, it is impermissible to rely on the hindsight afforded by the disclosure of the instant application to conclude that at the time the application was filed one of ordinary skill in the art would have reasonably believed that Cyr61 was a chemoattractant. *See* MPEP § 2141. In view of Yang merely speculating that Cyr61 is a chemoattractant, Applicant respectfully submits that the combination of cited references fail to provide the requisite expectation of success and, therefore, that the rejection of claims 4 and 6-8 under 35 U.S.C. § 103(a) is improper and should be withdrawn.

**2) The rejection of claim 24 under 35 U.S.C. § 103(a) as being obvious over Iwamoto in View of Yang and Lin**

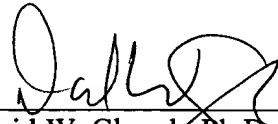
At page 3 of Paper 14, claim 24 remains rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Iwamoto, Yang and Lin, because Iwamoto allegedly teaches that basement membrane gels contain laminin and heparin sulfate proteoglycans, as well as other proteins. In view of Yang merely speculating that Cyr61 is a chemoattractant for the reasons set forth above, Applicant respectfully submits that the combination of cited references fail to provide the requisite expectation of success and, therefore, that the rejection of claim 24 under 35 U.S.C. § 103(a) is improper and should be withdrawn.

### CONCLUSION

In view of the above amendments and remarks, the Applicant respectfully submits that the instant application is in good and proper order for allowance. Early notification of this effect is solicited. If, in the opinion of the Examiner, a telephone conference would expedite this prosecution of the instant application, the Examiner is encouraged to call the undersigned at (312) 902-5464.

Respectfully submitted,

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## EXHIBIT A

Pursuant to 37 C.F.R. §1.121(b)(1)(iii), Applicant presents herewith marked-up text of the claims of this application as amended by the foregoing amendment.

Please replace the paragraph at page 16, lines 8-21 with the following paragraph:

The invention also extends to a method of screening for a modulator of cell migration comprising the steps of: (a) forming a gel matrix comprising Cyr61 and a suspected modulator of cell migration; (b) preparing a control gel matrix comprising Cyr61; (c) seeding endothelial cells capable of undergoing cell migration onto the gel matrix of step (a) and the control gel matrix of step (b); (d) incubating the endothelial cells; (e) measuring the levels of cell migration by inspecting the interior of the gel matrix and the control gel matrix for cells; (f) comparing the levels of cell migration measured in step (e), whereby a modulator of cell migration is identified by its ability to alter the level of cell migration in the gel matrix when compared to the level of cell migration in the control gel matrix. The endothelial cells include, but are not limited to, human cells, *e.g.*, human microvascular endothelial cells. The matrix may be formed from gelling materials such as [Matrigel] MATRIGEL®, collagen, or fibrin, or combinations thereof.

Please replace the paragraph at page 18, lines 15-26 with the following paragraph:

The invention also embraces an *in vivo* method of screening for a modulator of cell migration comprising the steps of: (a) removing a first central portion of a first biocompatible sponge and a second central portion of a second biocompatible sponge; (b) applying an ECM signaling molecule and a suspected modulator to the first central portion and an ECM signaling molecule to the second central portion; (c) reassociating the first central portion with said first biocompatible sponge and said second central portion with the second biocompatible sponge; (d) attaching a first filter to a first side of the first biocompatible sponge and a second filter to a second side of the first biocompatible sponge; (e) attaching a third filter to a first side of the second biocompatible sponge and a fourth filter to a second side of the second biocompatible sponge; (f) implanting each of the biocompatible sponges, each biocompatible sponge comprising the central portion and the filters, in a test animal; (g) removing each the sponge

following a period of incubation; (f) measuring the cells found within each of the biocompatible sponges; and (g) comparing the presence of cells in the first biocompatible sponge with the presence of cells in the second biocompatible sponge, whereby a modulator of cell migration is identified by its ability to alter the level of cell migration measured using the first biocompatible sponge when compared to the cell migration measured using the second biocompatible sponge. ECM signaling molecules include, but are not limited to, human Cyr61; the ECM signaling molecule may also be associated with [**Hydron**] **HYDRON®**. In addition, the *in vivo* method of screening for a modulator of cell migration may include the step of providing a radiolabel to the test animal and detecting the radiolabel in one or more of the sponges.

Please replace the paragraph at page 38, lines 18-28 with the following paragraph:

A strategy for the expression of recombinant *cyr61* was designed using a Baculovirus expression vector in Sf9 cells. Expression systems involving Baculovirus expression vectors and Sf9 cells are described in *Current Protocols in Molecular Biology* §§ 16.9.1-16.12.6 (Ausubel et al., eds., 1987). One embodiment of the present invention implemented the expression strategy by cloning the murine *cyr61* cDNA into pBlueBac2, a transfer vector. The recombinant clone, along with target AcMNPV (*i.e.*, *Autographa californica* nuclear polyhedrosis virus, or Baculovirus) DNA, were delivered into Sf9 cells by liposome-mediated transfection, using the [**MaxBac Kit**] **MAXBAC KIT®** (Invitrogen, Inc., San Diego, CA) according to the manufacturer's instructions. Recombinant virus was plaque-purified and amplified by 3 passages through Sf9 cells via infection.

Please replace the paragraph at page 39, line 9 to page 40, line 4 with the following paragraph:

Human *cyr61* was also expressed using the baculovirus system. A *Sma*I-*Hind*III fragment (corresponding to nucleotides 100-1649 of SEQ ID NO:3) of *cyr61* cDNA spanning the entire human *cyr61* open reading frame was subcloned into a pBlueBac3 baculovirus expression vector (Invitrogen). Recombinant baculovirus clones were obtained, plaque purified and amplified through three passages of Sf9 infection, using conventional techniques. Infection of Sf9 cells and human Cyr61 (hCyr61) purification was performed using standard techniques, with some modifications. Sf9 cells were maintained in serum-free Sf900-II medium (Sigma). Sf9



cells were seeded, at  $2-3 \times 10^6$  cells per 150 mm dish, in monolayer cultures and were infected with 5 plaque forming units (PFU) of recombinant virus per cell. The conditioned medium was collected at 8 and 96 hours post-infection, cleared by centrifugation ( $5000 \times g$ , 5 minutes) and adjusted to 50 mM MES [2-(N-Morpholino)ethanesulfonic acid], pH 6.0, 1 mM PMSF (phenylmethylsulfonyl fluoride), and 1 mM EDTA. The medium was mixed with [Sephacrose S] SEPHAROSE S® beads equilibrated with loading buffer (50 mM MES, pH 6.0, 1 mM PMSF, 1 mM EDTA, 150 mM NaCl) at a ratio of 5 ml [Sephacrose S] SEPHAROSE S® beads per 500 ml of conditioned medium and the proteins were allowed to bind to the [Sephacrose S] SEPHAROSE S® at 4°C (o/n) with gentle stirring. [Sephacrose S] SEPHAROSE S® beads were collected by sedimentation without stirring for 20 minutes and applied to the column. The column was washed with 6 volumes of 0.3 M NaCl in loading buffer and recombinant human Cyr61 was eluted from the column with a step gradient of NaCl (0.4-0.8 M) in loading buffer. This procedure resulted in 3-4 milligrams of purified Cyr61 protein from 500 ml of conditioned medium, and the purified Cyr61 was over 90% pure as judged by Coomassie Blue staining of SDS-gels.

Please replace the paragraph at page 48, lines 20-27 with the following paragraph:

Another criterion used to assess the properties of recombinant Cyr61 was its ability to bind heparin, described below. Purified recombinant murine Cyr61 bound quantitatively to heparin-SEPHAROSE® [sephacrose] at 0.15 M NaCl and was eluted at 0.8-1.0 M NaCl. This heparin binding capacity is similar to native murine Cyr61 obtained from serum-stimulated mouse fibroblasts. Because of the similarities of the murine and human Cyr61 proteins, recombinant human Cyr61 should exhibit properties similar to the native human Cyr61, as was the case for the murine polypeptides.

Please replace the paragraph at page 52, lines 3-14 with the following paragraph:

The heparin binding assay for native murine Cyr61, described in *Yang et al.*, was modified for the purified recombinant murine protein. Initially, recombinant purified Cyr61 was suspended in RIPA (Radioimmunoprecipitation assay) buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride). Next, 200 µl of a 50% (v/v) slurry of heparin-SEPHAROSE CL 6B® [Sephacrose CL 6B]

beads (Pharmacia-LKB Biotechnology, Inc., Piscataway, NJ) was added to 100 µl of the recombinant Cyr61 solution and incubated for 1 hour. Under these conditions, human Cyr61 was quantitatively bound to heparin-agarose. Application of a salt concentration gradient in RIPA buffer resulted in the elution of recombinant murine Cyr61 at 0.8-1.0 M NaCl. The elution profile of the recombinant protein was similar to the elution profile for native murine Cyr61.

Please replace the paragraph at page 58, lines 5-28 with the following paragraph:

Antibodies specifically recognizing Fisp12 have also been elicited using a fusion protein. The antigen used to raise anti-Fisp12 antibodies linked glutathione-S-transferase (GST) to the central portion of Fisp12 (GST-Fisp12), where there is no sequence similarity to Cyr61 (O'Brien and Lau, 1992). A construct containing cDNA encoding amino acids 165 to 200 of Fisp12 was fused to the glutathione-S-transferase (GST) coding sequence. This was done by using polymerase chain reaction (PCR) to direct synthesis of a fragment of DNA encompassing that fragment of *fisp12* flanked by a 5' *Bam*HI restriction site and a 3' *Eco*RI restriction site. The 5' primer has the sequence 5'-GGGGATCTGTGACGAGCCCAAGGAC-3' (SEQ ID NO:9) and the 3' primer has the sequence 5'-GGGAATTCGACCAGGCAGTTGGCTCG-3' (SEQ ID NO:10). For Cyr61-specific antiserum, a construct fusing the central portion of Cyr61 (amino acids 163 to 229), which contains no sequence similarity to Fisp12, to GST was made in the same manner using the 5' primer 5'-GGGGATCCTGTGATGAAGACAGCATT-3' (SEQ ID NO:11) and the 3' primer 5'-GGGAATTCAACGATGCATTTCTGGCC-3' (SEQ ID NO:12). These were directionally cloned into pGEX2T vector (Pharmacia-LKB, Inc.) and the clones confirmed by sequence analysis. The GST-fusion protein was isolated on glutathione [sepharose 4B] SEPHAROSE 4B® (Pharmacia-LKB, Inc.) according to manufacturer's instructions, and used to immunize New Zealand white rabbits. For affinity purifications, antisera were first passed through a GST-protein affinity column to remove antibodies raised against GST, then through a GST-Fisp12 or GST-Cyr61 protein affinity column to isolate anti-Fisp12 or anti-Cyr61 antibodies (Harlow *et al.*, [1988]).

Please replace the paragraph at page 63, line 15 to page 64, line 2 with the following paragraph:

$\alpha_v\beta_3$  integrin purification from HUVE cell lysates was done as described in *Pytela et al., Meth. Enzymol.*, 144:475-489 (1987). Briefly,  $10^8$  cells were lysed in 1 ml of PBS containing 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 0.5 mM PMSF and 100 mM octylglucoside. The lysate was passed four times through a 0.5 ml column containing RGDSPK [Sephacrose] SEPHAROSE® (prepared from the cyanogen bromide activated [Sephacrose] SEPHAROSE® CL 4B® as described in *Lam, S.C.-T., J. Biol. Chem.*, 267:5649-5655 (1992)). The column was washed with 10 ml of the lysis buffer and the bound protein was eluted with 2 ml of the same buffer containing 1 mM RGDS peptide at room temperature. The  $\alpha_v\beta_3$  integrin was dialyzed against PBS containing 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 5 mM octylglucoside and 0.1 mM PMSF with three changes of the dialysis buffer to remove the RGDS peptide. The protein was stored in aliquots at  $-70^\circ\text{C}$ . The purity of the integrin was determined by SDS-PAGE under non-reducing conditions, followed by silver staining. Western blotting with anti-CD47 antibody showed that this  $\alpha_v\beta_3$  integrin preparation does not contain any integrin-associated proteins.

Please replace the paragraph at page 72, lines 15-26 with the following paragraph:

The end product of *in vitro* angiogenesis is a well-defined network of capillary-like tubes. When cultured on gel matrices, *e.g.*, collagen, fibrin, or [Matrigel] MATRIGEL® gels, endothelial cells must first invade the matrix before forming mature vessels. ([Matrigel] MATRIGEL® is a complex mixture of basement membrane proteins including laminin, collagen type IV, nidogen/entactin, and heparan sulfate proteoglycan, with additional growth factors. *Kleinman et al., Biochem.* 25:312-318 (1986)). The invasive structures are cords which eventually anastomose to form the vessel-like structures. The angiogenic effect of human Cyr61 on confluent monolayers of human umbilical vein endothelial cells is assessed by seeding the cells onto three-dimensional collagen or fibrin gels, in the presence or absence of Cyr61. HUVE cells do not spontaneously invade such gels but do so when induced by agents such as tumor promoters.

Please replace the paragraph at page 79, lines 8-25 with the following paragraph:

An *in vivo* assay for endothelial cell migration has also been developed. In general, the assay protocol is consistent with the disclosure of *Tolsma et al.*, 1993. To assess angiogenesis associated with the formation of granulation tissue (*i.e.*, the newly formed, proliferative, fibroblastic dermal tissue around wounds during healing), sponge implants were used as previously described (*Fajardo, et al., Lab. Invest.* 58:718-724 [1988]). Polyvinyl-alcohol foam discs (10-mm diam x 1-mm thick) were prepared by first removing a 2-mm diameter central core of sponge. PBS or an RGDS peptide (other possible test compounds include fragments of Cyr61, RGDS peptide, small molecules such as mannose-6-phosphate) at 100  $\mu$ M were added to the sponge core which was then coated with 5  $\mu$ l of sterile [**Hydron**] **HYDRON®** (Interferon Sciences, New Brunswick, NJ). After solidifying, the coated core was returned to the center of the sponge which was then covered on both sides with 5  $\mu$ m filters and secured in place with glue (Millipore Corp., Bedford, MA). One control and one test disc were then implanted subcutaneously in the lower abdomen of anesthetized Balb/c female mice where granulation tissue could invade the free perimeter of the disc. Wounds were closed with autoclips and animals left undisturbed until sacrificed.

Please replace the paragraph at page 88, lines 7-14 with the following paragraph:

To provide slow release of the protein after implantation in the cornea, protein is mixed with poly-2-hydroxyethylmethacrylate ([**Hydron**] **HYDRON®**), or an equivalent agent, to form a pellet of approximately 5  $\mu$ l. Implants made in this way are rehydrated with a drop of sterile lactated Ringers solution and implanted as described above. After implantation, the corneal pocket is sealed with erythromycin ointment. After implantation, the protein-[**Hydron**] **HYDRON®** pellet should remain near the limbus of the cornea (cornea-sclera border) and vision should not be significantly impaired.

Please replace the paragraphs at page 89, lines 3-29 with the following paragraphs:

Each protein is typically tested in three doses, in accordance with the practice in the art. Those of ordinary skill in the art realize that six positive corneal responses per dose are required to support an identification of an angiogenic response. An exemplary cornea assay includes three doses of the protein under study, with six rats being tested at each dose. Additionally, six

animals are exposed to a buffer-[Hydron] HYDRON® implant and serve as negative controls. Exposure of at least three animals to a known angiogenic factor-[Hydron] HYDRON® implant serve as positive controls. Finally, to demonstrate the specificity of any observed response, six animals are exposed to implants containing a single dose of the protein under study, an excess of neutralizing antibody, and [Hydron] HYDRON®.

A cornea assay as described above was performed to assess the ability of Cyr61 to induce angiogenesis. Four animals were given negative control implants containing a buffer-[Hydron] HYDRON® (both eyes). Each of these animals failed to show any blood vessel development in either eye after seven days. Six animals received implants containing a biologically effective amount of Fibroblast Growth Factor (0.15  $\mu$ M) in one eye and a control pellet in the other eye; all six showed angiogenic development in the eye receiving FGF, none showed neovascularization in the eye receiving the negative control. Seven animals received 1  $\mu$ g/ml Cyr61, in one eye and all seven of these eyes showed blood vessel growth; one of the seven eyes receiving a negative control showed angiogenic development. Finally, four animals received implants locally releasing 1  $\mu$ g/ml Cyr61 ([Hydron] HYDRON® prepared with a 10  $\mu$ g/ml Cyr61 solution) and a specific anti-Cyr61 antibody in three-fold excess over Cyr61; none of the eyes of this group showed any angiogenic development. Thus, the in vivo assay for angiogenesis identifies angiogenic factors such as FGF and Cyr61. The assay also is able to reveal inhibition of angiogenic development induced ECM signaling molecules such as Cyr61.

Please replace the paragraph at page 110, lines 9-15 with the following paragraph:

Recombinant Cyr61 and mutant Cyr61, synthesized in a Baculovirus expression system using Sf9 insect cells, were purified from serum-free conditioned media by chromatography on [Sepharose S] SEPHAROSE S® columns. The purity and yield of the proteins were determined by SDS-PAGE followed by Coomassie blue staining and immuno- blotting. Human fibronectin, human vitronectin, rat tail Type-I collagen and mouse laminin were obtained from Collaborative Research, MA.

Please replace the paragraph at page 116, line 23 to page 117, line 22 with the following paragraph:

Mutant Cyr61 proteins deficient in heparin binding were generated to examine the effect of such changes on fibroblast adhesion. Conventional site-directed mutagenesis techniques were used to produce mutant Cyr61 polypeptides having altered heparin-binding motifs. Two putative heparin-binding motifs were found within the carboxyl-terminal domain in Cyr61 that conform to the consensus XBBXB sequence for heparin binding (where B denotes basic amino acid residues such as lysine or arginine). Site-directed mutagenesis was used to replace the lysine and arginine residues in the motifs with alanine, thus creating two Cyr61 variants (H1 and H2) each having one of the two heparin binding motifs mutated. In addition, both motifs were mutated in a Cyr61 double mutant (DM) variant. A comparison of the mutated amino acid sequence H<sub>2</sub>N-SLKAGAACSATAKSPEPVRFTYAGCSSVAAYAPKYCG-CO<sub>2</sub>H (SEQ ID NO:30) with residues 278-314 of SEQ ID NO:2 (wild-type mouse Cyr61), shows clusters of amino acid changes between residues 280-290 (H1, underscored above) and between residues 305-310 (H2, underscored above); both sets of clustered changes are found in DM. These mutations were created using the full-length *cyr61*; the mutant constructs were expressed in, and purified from, recombinant Baculovirus-transformed insect cells. Equal amounts of conditioned media of insect SF9 cells infected with Baculovirus expressing wild-type or mutant Cyr61 protein were loaded on CL-6B Heparin [Sephacrose] SEPHAROSE® columns. After washing with 20 bed volumes of RIPA buffer, bound protein was eluted with RIPA buffer containing increasing concentrations of sodium chloride. Equal amounts of eluate from each fraction were analyzed on SDS-PAGE gels followed by Western blotting to visualize Cyr61 protein. Antibodies used were rabbit polyclonal antibodies against bacterial GST-Cyr61. The H1 mutant Cyr61 polypeptide eluted over the range of 0.4-0.8 M NaCl; H2 eluted over the range 0.4-1.0 (primarily between 0.6-0.8) M NaCl; DM eluted during the washing and up to 0.25 M NaCl; and wild-type Cyr61 eluted at 0.8-1.0 M NaCl. These elution profiles indicate that H1 and H2 exhibited somewhat decreased heparin-binding affinities, whereas DM was severely deficient in heparin binding

Please replace the paragraph at page 127, lines 3-20 with the following paragraph:

Southern blotting was performed as described in "Current Protocols in Molecular Biology" (*Ausubel et al.*, [1999]). Briefly, *Eco*RI fragments of genomic DNA were fractionated

by electrophoresis through 0.8% agarose gels and blotted onto nylon membranes (Bio-Rad) by downward capillary transfer with alkaline buffer (0.4 M NaOH). The probes, a *Bam*HI-*Eco*RI fragment 3' to the long arm of the targeting construct (p61geo) or the *neo* coding region sequences, were prepared by random primer labeling (Prim-it II, Stratagene) using [ $\alpha$ - $^{32}$ P] dCTP (NEN). Membranes were prehybridized in hybridization buffer (7% SDS, 0.5 M NaHPO<sub>4</sub> (pH 7.0), and 1 mM EDTA) at 65°C for 15 minutes in a rolling bottle. Fresh hybridization buffer was added with the probe and membranes were hybridized for 18 hours. Hybridized membranes were briefly rinsed in 5% SDS, 40 mM NaHPO<sub>4</sub> (pH 7.0), 1 mM EDTA and then washed for 45 minutes at 65°C with fresh wash solution. This wash solution was replaced with 1% SDS, 40 mM NaHPO<sub>4</sub> (pH 7.0), 1 mM EDTA and washed twice for 45 minutes at 65°C with fresh solution. After washing, membranes were exposed to a screen, which was then scanned using a [PhosphorImager] **PHOSPHORIMAGER®** (Molecular Dynamics). Blots were routinely stripped and re-probed with the control *neo* probe to ensure that random integration had not occurred, using conventional techniques.

Please replace the paragraph at page 127, line 27 to page 128, line 4 with the following paragraph:

Genotyping was also done by PCR using a [RoboCycler] **ROBOCYCLER®** (Stratagene). Primers were designed to amplify a 2.1 kb DNA fragment from mutant alleles. The PCR product covers the 5'-flank of the short arm of the targeting construct through to the sequence of *lacZ* ( $\beta$ -gal) within the targeting construct. The upper PCR primer sequence was 5'-CACAACAGAAGCCAGGAACC-3' (SEQ ID NO:24) and the lower PCR primer sequence was 5'-GAGGGGACGACGACAGTATC-3' (SEQ ID NO:25). PCR reaction conditions were 95°C for 40 seconds, 63°C for 40 seconds, and 68°C for one minute, for 35 cycles.

Please replace the paragraph at page 131, line 29 to page 132, line 12 with the following paragraph:

Defects in placental development, a major cause of embryonic death in *cyr61*<sup>-/-</sup> mice, were further analyzed. Histological analyses of mouse placentae generally followed *Suri et al.*, (1998). Briefly, placentae from E12.5 embryos were dissected in cold PBS and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4°C for overnight. Fixed placentae were

then dehydrated through increasing concentrations of alcohol (50%, 75%, 90%, 95%, and 100%) two times. Dehydrated tissue was then cleared with [**Hemo-De**] **HEMO-DE®** (a xylene alternative), 1:1 ethanol/[**Hemo-De**] **HEMO-DE®** (Fisher), and 100% [**Hemo-De**] **HEMO-DE®**, and the clearing process was repeated. Cleared tissues were then equilibrated in a 1:1 mixture of paraffin: [**Hemo-De**] **HEMO-DE®** at 60°C for one hour in a vacuum oven and the process was repeated. Tissues were embedded in paraffin with Histoembedder (Leica). The paraffin-embedded placentae were cut into 10 µm slices with a microtome (Leica). Finally, tissue sections were subjected to Harris' Hematoxylin and Eosin staining (*Asahara et al., Circ. Res.* 83:233-240 [1998]).

Please replace the paragraph at page 133, line 22 to page 134, line 5 with the following paragraph:

X-gal staining was also used to assess embryonic development in various *xyr61* backgrounds. (The targeting DNA, *p61geo*, was designed to knock out the *Cyr61* gene and also to "knock in" a  $\beta$ -gal gene as a marker to reflect the expression of *Cyr61*). X-gal (*i.e.*, 5-Bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside) staining for  $\beta$ -galactosidase expression was performed on heterozygous *cyr61*<sup>+/-</sup> embryos staged from E9.5 to E11.5. The staining was done as described (*Suri et al.*, [1998]). Staged embryos were fixed in a 0.2% paraformaldehyde solution at 4°C overnight. Fixed tissue was incubated in 30% sucrose in PBS plus 2 mM MgCl<sub>2</sub> at 4°C overnight. Tissue was then embedded in OCT on dry ice and cut with a cryotome into 7 µm sections. Frozen tissue sections were post-fixed in 0.2% paraformaldehyde and stained with X-gal (1 mg/ml) at 37°C for 3 hours in the dark. Slides were counter-stained with 1% Orange G. Stained slides were then serially dehydrated through increasing concentrations of methanol, cleared with [**Hemo-De**] **HEMO-DE®**, and slides were mounted.

Please replace the paragraph at page 136, lines 7-15 with the following paragraph:

Recombinant *Cyr61* and *Fisp12/mCTGF*, synthesized in a Baculovirus expression system using Sf9 insect cells, were purified from serum-free conditioned media by chromatography on [**Sepharose S**] **SEPHAROSE S®** as described (*Kireeva et al.*, [1997]; *Kireeva, et al.*, [1996]). SDS-PAGE analysis of purified *Cyr61* and *Fisp12/mCTGF* revealed the presence of single Coomassie Blue-stained bands of 40-kDa and 38-kDa, respectively. On immunoblots, the



purified proteins reacted specifically with their cognate antibodies. Protein concentrations were determined using the BCA protein assay (Pierce) with bovine serum albumin (BSA) as the standard.

Please replace the paragraph at page 136, line 27 to page 137, line 10 with the following paragraph:

Microtiter wells (~~[Immulon 2 removawell strips]~~ **IMMULON 2 REMOVAWELL STRIPS®**, Dynex Technologies, Inc.) were coated with Cyr61, Fisp12/mCTGF, or fibrinogen (25 µg/ml, 50 µl/well) incubated overnight at 22°C, and then blocked with 3% BSA at 37°C for 2 hours. Washed platelets were added to the wells (100 µl/well) in the presence and absence of platelet agonists and incubated at 37°C for 30 minutes. The wells were washed with HEPES-Tyrode's buffer and adherent platelets were detected with <sup>125</sup>I-mAb15, an anti-β<sub>3</sub> monoclonal antibody. Exposure to the labeled antibody (50 nM, 50 µl/well) proceeded for 1 hour at 22°C. After extensive washing with HEPES-Tyrode's buffer, bound radioactivity was determined by γ-counting. In inhibition studies, washed platelets were pre-incubated with blocking peptides or antibodies at 37°C for 15 minutes prior to addition to microtiter wells. In experiments to examine the effect of divalent cation chelation, EDTA (5 mM) was added to suspensions of washed platelets and pre-incubated at 37°C for 15 minutes.

Please replace the paragraph at page 138, lines 9-21 with the following paragraph:

To further substantiate the activation-dependent adhesion of platelets to these proteins, an acid phosphatase assay designed to quantitate the relative numbers of adherent platelets was performed. This assay measured the acid phosphatase activity of adherent platelets. Following the adhesion and washing procedures described above, the substrate solution (0.1 mM sodium acetate, pH 5.0, 20 mM *p*-nitrophenylphosphate, and 0.1% [**Triton X-100**] **TRITON X-100®**; 150 µl/well) was added and incubated for 2 hours at 37°C. The reaction was stopped by the addition of 20 µl 2N NaOH, and absorbance at 405 nm was measured. Both the <sup>125</sup>I-mAb15 binding assay and the acid phosphatase assay for adhesion of ADP-stimulated platelets to fibrinogen, Fisp12/mCTGF, and Cyr61, produced similar results. Because the amounts of bound <sup>125</sup>-mAb15 were directly proportional to the quantity of integrin α<sub>IIb</sub>β<sub>3</sub> on the adherent platelets, the acid phosphatase assay was used in subsequent studies.

Please replace the paragraphs at page 140, line 11 to page 141, line 3 with the following paragraphs:

A solid-phase binding assay to detect the receptor-ligand interactions showed that  $\alpha_{IIb}\beta_3$  binds directly to Fisp12/mCTGF and Cyr61. In these experiments, activated and unactivated  $\alpha_{IIb}\beta_3$  were purified from platelet lysates. Activated  $\alpha_{IIb}\beta_3$  was purified by RGD affinity chromatography, as described (*Knezevic et al., J. Biol. Chem.* 271:16416-16421 [1996]). Briefly, outdated human platelets were isolated by differential centrifugation and solubilized in lysis buffer (10 mM HEPES, pH 7.4, 0.15 M NaCl, containing 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 mM N-ethylmaleimide, and 50 mM octylglucoside). The octylglucoside extract was incubated with 1 ml GRGDSPK-coupled [**Sepharose 4B**] **SEPHAROSE 4B®** overnight at 4°C. After washing with 15 ml column buffer (same as lysis buffer except it contained 25 mM octylglucoside), bound  $\alpha_{IIb}\beta_3$  was eluted with 1.7 mM  $\text{H}_{12}$  (2 ml) in column buffer. The  $\text{H}_{12}$  eluate was applied to a [**Sephacryl**] **SEPHACRYL®** S-300 High Resolution column (1.5 x 95 cm), and  $\alpha_{IIb}\beta_3$  was eluted with 10 mM HEPES, pH 7.4, 0.15 M NaCl, 1 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$  and 25 mM octylglucoside.

Unactivated  $\alpha_{IIb}\beta_3$  was isolated by the method of *Fitzgerald et al., Anal. Biochem.* 151:169-177 (1985), with slight modifications. The flow-through fraction of the GRGDSPK-[**Sepharose**] **SEPHAROSE®** column was applied to a concanavalin A-[**Sepharose 4B**] **SEPHAROSE 4B®** column (1 x 20 cm). Unbound proteins were washed with 50 ml column buffer, and bound  $\alpha_{IIb}\beta_3$  was then eluted with 100 mM mannose dissolved in column buffer. Fractions containing  $\alpha_{IIb}\beta_3$  were further purified on a [**Sephacryl**] **SEPHACRYL®** S-300 High Resolution column.